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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

Docket Number	27139	Type a plus sign (+) inside this box ->	+

INVENTOR(s) / APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
AMIT	Michal		Misgav, Israel
ITSKOVITZ-ELDOR	Joseph		Haifa, Israel

TITLE OF THE INVENTION (280 characters max)

STEM CELLS AND EMBRYONIC BODIES CARRYING DEFECTIVE GENES AND USE OF SAME OR TISSUES OR ORGANS DEVELOPED THEREFROM AS IN VITRO OR IN VIVO EXPERIMENTAL MODELS FOR DEVELOPING CURES FOR GENETIC DISEASES

CORRESPONDENCE ADDRESS

G. E. EHRLICH (1995) LTD.
c/o ANTHONY CASTORINA
2001 JEFFERSON DAVIS HIGHWAY
SUITE 207

STATE	VIRGINIA	ZIP CODE	22202	COUNTRY	USA

ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ **Specification**	*Number of Pages*	21	☑ **Applicant is entitled to Small Entity Status**
☑ **Drawing(s)**	*Number of Sheets*	6	☑ **Other** (specify) 3 CLAIMS

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☐ A check or money order is enclosed to cover the filing fees ☑ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 50-1407	**FILING FEE AMOUNT ($)**	**$ 80.-**

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

☐ Yes, the name of the US Government agency and the Government contract number are: ________________

Respectfully submitted,

SIGNATURE ______________________ Date **November 27, 2003**

25,457
REGISTRATION NO.
(if appropriate)

TYPED or PRINTED NAME **SOL SHEINBEIN**

☐ Additional inventors are being named on separately numbered sheets attached hereto

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

STEM CELLS AND EMBRYONIC BODIES CARRYING DEFECTIVE GENES AND USE OF SAME OR TISSUES OR ORGANS DEVELOPED THEREFROM AS IN VITRO OR IN VIVO EXPERIMENTAL MODELS FOR DEVELOPING CURES FOR GENETIC DISEASES

Inventors: Michal Amit and Joseph Itskovitz-Eldor

Abstract

The pluripotency of human embryonic stem (hES) cells renders them ideal candidates for the research on the nature and course of specific chronic diseases. hES cell lines harboring genetic diseases may be derived by either genetic manipulation of existing cell lines or isolation of new lines from embryos which are already genetically compromised. A potential source of genetically-compromised embryos are those which were analysed in the pre-implantation genetic diagnosis (PGD) program, and diagnosed as such.

In the present study we report the derivation of hES cell lines from surplus PGD embryos. Five lines were derived and found to possess the typical features of hES cells. One of these lines harboured the Van Waardenburg disease (deletion at the PAX3 gene) while an additional one was detected as Myotonic Dystrophy carrier (a $(CTG)_n$ a repeated expansion mutation in the 3'-untranslated region (3'-UTR) of the myotonic dystrophy protein kinase gene). The remaining cell lines were carriers of recessive mutations, and were therefore regarded as healthy.

Models established by the use of hES cell lines carrying specific genetic defects may be highly effective in the development of drug or gene therapies designed to treat these diseases, and for research aiming at gaining better understanding of the mechanisms underlying these diseases.

Key words: embryo, embryoid bodies, embryonic stem cells, preimplantation genetic diagnosis, Myotonic dystrophy, Van Waardenburg syndrome, PAX3.

Introduction

Human ES (hES) cells are pluripotent cells derived from the inner cell mass (ICM) of the preimplanted blastocyst. In addition to their broad developmental potential, ES cells are also characterized by their ability for prolonged undifferentiated proliferation while maintaining normal karyotypes. Due to these unique features, hES cells may be used in a wide range of research applications in fields such as development and differentiation processes, lineage commitment, self-maintenance and maturation of progenitor cells . They may also serve as research tools for the study of gene and protein function and drug testing. In the future these cells may be utilized for cell-based therapies.

The first hES cell lines were derived in 1998 from the inner cell masses (ICMs) of surplus blastocysts donated by couples undergoing IVF treatments (Thomson et al, 1998). Here we show non-retrieved embryos from the pre-implantation genetic diagnosis (PGD) program as a possible source of donated embryos for hES cell isolation. During the PGD process, one out of 6-8 cells of a three-day-old embryo is removed and analyzed for the existence of genetic defects either by Polymerase Chain Reaction (PCR) or by fluorescence in-situ hybridization (FISH). The procedure allows couples who are carriers of genetic diseases to examine their embryos before implantation and to retrieve the healthy embryos only.

The use of PGD-donated embryos facilitates the isolation of ES cell lines harboring different genetic defects. The resultant lines may be then utilized for research on the mechanism underlying the disease and the development of treatment strategies.

In the present study we report the derivation of two ES cell lines harbouring genetic disease. The first carries Myotonic Dystrophy (DM) and the other harbours the Van Waardenburg Syndrome (WS). DM or Steinert's disease is a progressive autosomal dominant disease characterized by increasing muscle weakness, myotonia, cataracts, and endocrine abnormalities such as diabetes and testicular atrophy (Mankobi and Thornton, 2002). The disease is found in 2.1 to 14.3 newborns out of 100,000 deliveries worldwide (Meola, 2000). The gene for DM was cloned and the mutation was shown to be an expanded trinucleotide $(CTG)_n$ repeats DM1 or $(CCTG)_n$ repeats DM2 (Brook et al, 1992; Liquori et al, 2001). The WS is an autosomal dominant disease featured by deafness, pigmentary anomalies, "dystopia canthorum" (lateral displacement of the inner canthi of the eyes), heterochromia irides, and synophrys (McKusick 1992; Waardenburg, 1951). The incidence prevalence was estimated to be 1.44 to 2.05 newborns out of 100,000 deliveries worldwide (Fraser 1976). A polymerase chain reaction (PCR)- based assay allows diagnosis of these diseases. The characteristics of the ES cell lines harbouring these genetic diseases are discussed.

Materials and methods

Blastocyst cultivation:

Discarded zygotes were donated by couples undergoing PGD at Rambam Medical Center who signed consent forms. The couple underwent the traditional IVF procedure with ovarian stimulation by gonadotrophins and oocyte retrieval. Fertilization was performed by sperm injection into the oocytes (ICSI) to prevent DNA contamination by sperm attachment to the zona pellucida as often occurs in IVF. Fertilization was checked 18-19 hours post ICSI for pronuclear formation and zygotes with normal pronucleai were

transferred to growth medium for further development. Embryos that had reached 6-8 cells on day three post fertilization were studied by past blastomere biopsy for PGD analysis.

Zygotes were cultured to the blastocyst stage according to our IVF laboratory standard protocol: drops under oil using specialized Cook media for insemination, growth and blastocyst stage ?? (Cook - IM, GM and BM, respectively, Queensland, Australia).

Blastomere biopsy:

Blastomere biopsy was performed using a micromanipulation procedure on all embryos that reached 6-8 cells. Each embryo was gently held by a holding micropipette (20 micron diameter aperture). An aperture micropipette of 10-micron in diameter filled with acid Tyrode's solution (pH 2.4; Sigma Chemical Co., St. Louis, MO, USA) was used to drill the zona pellucida. The size of the opening created was slightly smaller than that of the blastomere (~40 microns). A 40-micron micropipette filled with medium was inserted through this opening, and the nearest blastomere(s) was aspirated. Each blastomere was then individually transferred to a PCR tube for analysis.

Pre-implantation genetic diagnosis for Myotonic Dystrophy:

The selected blastomere? was transferred to a PCR tube containing 2μl of 125 μg/ml PCR grade proteinase K (Roche Diagnostic GmbH, Mannheim, Germany) and 1μl of 17μM SDS (Sigma Chemical Co., St. Louis, MO, USA) prepared in nuclease free water (Promega, Madison WI). Cells underwent lysis by incubation for one hour in 37 °C followed by heat inactivation for 15 min in 95°C. Following the lysis of the cells, 17μl of the first round PCR mixture were added. For the nested PCR reaction, 2μl of the first PCR round were diluted into 20μl total PCR reaction. For myotonic dystrophy we used the protocol developed by Sermon and colleagues (Sermon et al, 1997).

Following lysis of the cells with proteinase k and SDS, reaction mixture was added to the cells to a final volume of 20 μl and final concentrations of 10% DMSO, 2mM MgCl2, 0.2mM dNTP, 2pmole of each primer and 1IU BioTaq polymerase (Bioline Ltd, London, UK) in the manufacturer's recommended buffer. The outer primers 101 and 102 were as described by Brook et al 1992. PCR was carried out using the following programme: 5 min denaturation at 95°C, followed by 35 cycles of 30 s at 95, 30 s at 65°C and 30 s at 72°C, followed by 7 min at 72°C. The primers for the nested PCR were 409 and 410 (Mahadeven et al., 1992). Two microliters from the first PCR were used as a template to a final volume of 20 μl and final concentrations of 1.5mM MgCl2, 0.2mM dNTP, 2pmole of each primers and 1IU Taq polymerse (Qiagen GmbH, Hilden, Germany) in the manufacturer recommended buffer with the addition of Q-solution (Qiagen according to the manufacturer recommendation). The same PCR conditions were used except for the denaturation temperature which was reduced to 94°C.

The PCR products were separated on a 3% nusieve agarose 3:1 (Biowhittaker Molecular Applications, Rockland, ME USA).

The patients involved were informed for only one allele (one of the father's allele was similar in size to the mother allele). Following PGD, only those embryos that exhibited two alleles (one from each parent) were retrieved. Those which were not retrieved were donated. To further confirm the number of DM protein kinase (CTG)n repeats , the PCR products were separated on 8% polyacrylamide denatured gel and visualized using silver staining (Lerer et al, 1994). As controls we used PCR products of affected and normal persons. Various sizes of $(CTG)_n$ repeats were used as size markers.

<u>Pre-implantation genetic diagnosis for the Van Waardenburg syndrome:</u>

The mutation was previously published in Human Mutation 3:205 in 1994. The family number is BU-53 and the mutation is a deletion of 28 bp at the 3' of exon 2. The primers pax3f (CTTCCCACAGTGTCCACTCC) and pax3r (GAGGATTGCAAGGCTTATGG) were used for the first PCR cycle. Following lysis of the cells with proteinase k and SDS, reaction mixture was added to the cells to a final volume of 20 μl and final concentrations of 1.5mM MgCl2, 0.2mM dNTP, 2pmole of each primers and 1IU BioTaq polymerase (Bioline Ltd, London, UK) in the manufacturer's recommended buffer. PCR was carried out using the following programme: 5 min denaturation at 95°C, followed by 35 cycles of 30 s at 95, 30 s at 60°C and 30 s at 72°C, followed by 7 min at 72°C. The primers NL1 (ACGGCAGGCCGCTGCCCAAC) and HUP2 (AGTCTGGGAGCCAGGAG) were used for the nested PCR. Two microliters from the first PCR were used as a template to a final volume of 20 μl and final concentrations of 1.5mM MgCl2, 0.2mM dNTP, 2pmole of each primers and 1IU Taq polymerse (Qiagen GmbH, Hilden, Germany) in the manufacturer's recommended buffer with the addition of Q-solution (Qiagen according to the manufacturer's recommendation). The same PCR conditions were used except for the denaturation temperature which was reduced to 94°C.

The PCR products were separated on a 3% nusieve agarose 3:1 (Biowhittaker Molecular Applications, Rockland, ME USA). Since the syndrome is dominant, the affected parent and embryos exhibited two alleles.

Derivation of hES cell lines:

After digestion of the zona pellucida by Tyrode's acidic solution (Sigma, St Louis, MO, USA) or its mechanical removal, the exposed blastocysts were placed in whole on a mitotically inactivated mouse embryonic fibroblast (MEF) feeder layer. For the derivation and initial passages, cells were grown in a culture medium consisting of 80% KO-DMEM,

and supplemented with 20% defined FBS (HyClone, Utah, USA), 1mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock (all from Gibco Invitrogen corporation products, San Diago, CA, USA products). The cells were initially passaged mechanically every four to ten days.

Culture of hES cells:

From passage 7-10 and onward, the cells were cultured with MEFs using culture medium consisting of 85% KO-DMEM, and supplemented with 15% ko-serum replacement, 1mM L-glutamine, 0.1 mM β–mercaptoethanol, 1% non-essential amino acid stock., and 4ng/ml basic fibroblast growth factor. The cells were passaged routinely every four to six days using 1mg/ml type IV collagenase (All products from Gibco Invitrogen corporation products, San Diago, CA, USA). The cells were frozen in liquid nitrogen using a freezing solution consisting of 10% DMSO (Sigma, St Louis, MO, USA), 10% FBS (Hyclone, Utah, USA) and 80% KO-DMEM.

Karyotype analysis:

The karyotype analysis was performed as previously described (Amit et al, 2003). For cell division block in mitotic metaphase we used colcemid-spindle formation inhibitor (karyoMax colcemid solution, Gibco Invitrogen corporation products, USA). Nuclear membranes were broken after hypotonic treatment. For the chromosome visualization we used G-band standard staining (Giemsa, Merck, Darmstadt, Germany). The karyotypes were analyzed and reported according to the "International System for Human Cytogenetic Nomenclature" (ISCN). At least 20 cells were examined from each sample, two samples from each line.

EB formation:

For the formation of EBs four to six confluent wells were used in a six-well plate (40-60 c^2m). ES cells were removed from their culture dish using 1mg/ml type IV collagenase, further broken into small clumps using 1000 µl Gilson pipette tips, and cultured in suspension in 58 mm Petri dishes (Greiner, Germany). EBs were grown in medium supplemented with 20% FBSd (HyClone, Utah, USA) instead of SR, without the addition of bFGF.

Immunostaining:

hES cells were fixed with 4% paraformaldehyde, and exposed to the primary antibodies (1:20) overnight at 4 °C. As secondary antibodies (1:100) we used Cys 3 conjugated antibodies (Chemicon International, Temecula CA, USA). As primary antibodies we used stage-specific embryonic antigen (SSEA) 1,3 and 4 (Hybridoma bank, Iuwa, USA), and tumour recognising antigen (TRA) 1-60 and TRA1-81, which were kindly provided by Prof. P. Andrews from the University of Sheffield, UK.

Teratoma formation:

Cells from six confluent wells in a six-well plate (60 c^2m) were harvested and injected into the rear leg muscles of four-week-old male SCID-beige mice. 12 weeks post injection the resultant teratomas were fixed in 10% neutral-buffered formalin, dehydrated in graduated alcohol (70%-100%) and embedded in paraffin. For histological examination, 1-5 µm sections were deparafined and stained with hematoxylin/eosin (H&E).

RT-PCR:

Total RNA was isolated from cells grown for 34 and 41 passages post derivation, or 10 day-old EBs using Tri-Reagent (Sigma, St. Louis, MO), according to the manufacturer's

recommended protocol. cDNA was synthesized from 1 μg total RNA using MMLV reverse transcriptase RNase H minus (Promega, Madison, WI, USA). PCR reaction included denaturation for 5 minutes in 94°C followed by repeated cycles of 94°C for 30 seconds, annealing temperature (as in Table 1) for 30 seconds and extension at 72°C for 30 seconds. PCR primers and reaction conditions used are described in Table 1. PCR products were size-fractionated using 2% agarose gel electrophoresis.

Results

Seventy six discarded embryos were donated by the PGD program at Rambam Medical Centre; the donor couples signed consent forms which were approved by the hospital and national health committee. The donated embryos were either carrying the genetic diseases tested, or discarded embryos which were found unsuitable for embryo transfer according to the IVF grading, or embryos that underwent PGD with unclear results, whose parents decided to not retrieve. Out of these 76, 31 developed into the blastocyst stage (41%)(Fig. 1a). One of the embryos was early blast on day 6 post fertilization. For ES cell line isolation, embryos were plated in whole (n=32). An ICM outgrowth (Fig 1b) was detected in five of the embryos plated in whole, from which five pluripotent cell line were isolated (5/32, 15.6%). Table 2 summarizes the development of the embryos.

The 5 lines obtained were further tested for genetic diseases. Two lines were found to be heterozygous for the cystic fibrosis mutation (W1282) and the metachromatic leukodystrophy disease mutation (recessive autosomal diseases). One line was homozygous for healthy alleles of gorlin disease (data not shown), and two lines were found to harbour DM and WS.

To confirm the existence of the WS and DM mutations in the resultant lines, DNA from these lines was examined using methods similar to those of the PGD analysis. The

results are demonstrated in Fig 2. The PCR products of the WS line resulted in the same pattern of separation on a 3% nusieve agarose as the affected parent (Fig. 2a). To further confirm this finding we isolated the PCR products from the gel and had them undergo sequencing analysis. The sequencing results demonstrated that both the WS line and the affected parent harbour the same deletion of 28 bp at the 3' of exon 2. The PCR conducted for the PGD analysis of DM, revealed that the embryo did not carry the healthy alleles of the parents. In order to analyze the resulted DM pluripotent cell line, the PCR products of the line were separated on 8% polyacrylamide denatured gel and visualized using silver staining. The results confirmed the existence of $(CTG)_n$ repeats typical of affected persons (Fig. 2b).

The lines harboring genetic diseases were closely examined for ES cell line characteristics. Both lines demonstrated colonies and cell morphology typical of human ES cell lines, i.e. round colonies with clear borders, spaces between cells, high cytoplasm to nucleus ratio and existence of two to four nucleoli (Fig 1 c-d). Immunostaining using clonal primary antibodies for undifferentiated surface markers revealed negative staining for stage-specific embryonic antigen (SSEA)-1, weak or no staining for SSEA3, and positive staining for SSEA4, tumor recognition antigen (TRA)-1-60 and TRA-1-81 as previously shown for human ES cell lines (Thomson at el, 1998; Reubinoff et al, 2000). Examples of the immunostaining results are illustrated in Fig. 3.

Karyotype analysis was conducted on cells at passage 30 and 17 for the SW and DM cell lines, respectively. All tested cells exhibited normal 46, XX karyptypes.

The pluripotency of the affected cell lines was tested both *in vitro* and *in vivo*. As other human ES cells (Itskovitz-Eldor et al, 2000), when cultured in suspension both cell lines spontaneously formed embryoid bodies (EBs), including cystic EBs (Fig. 4 a-b). Within these EBs the cells spontaneously differentiated into representative cell types of the

three embryonic germ layers as demonstrated both by immunostaining with specific markers (Fig. 4 c-f) and by PCR analysis for specific genes (Fig. 5). Following their injection into the hindlimb muscle of SCID Beige mice, both cell lines created teratomas including representative tissues of the embryonic germ layers such as cartilage and muscle tissue (mesoderm), gut-like epithelium (endoderm), and nerve tissue (ectoderm). Examples are illustrated in Fig. 6.

The three cell lines found healthy were also examined for the main ES cell line features. These lines were shown to exhibit normal karyotypes, to spontaneously form EBs while cultured in suspension, and teratomas following injection into SCID mice (data not shown).

Discussion

We present here newly derived human pluripotent cell lines, resulting from surplus PGD embryos , which exhibit the most important features of human embryonic stem cells: expression of typical undifferentiated surface markers, stable and normal karyotypes, and developmental potential to differentiate into representative cell types of the embryonic germ layers both *in vitro* and *in vivo* (Thomson et al, 1998; Reubinoff et al, 2000). Thus, non-retrieved embryos from the PGD program, similarly to discarded embryo from the IVF program (Mitalipova et al, 2003) can be used as an additional source for human embryos for the isolation of human ES cell lines.

Relatively to other reports on human ES cell line derivation which detailed success rates of 30 to 60 percent, the line isolation successes rates in this study (15.6%, 5/32) are low (Thomson et al, 1998; Reubinoff et al, 2000; Amit and Itskovitz-Eldor 2002; Mitalipova et al, 2003). One of the possible reasons for the reduced number is the fracture in the ZP. The drill in the ZP performed for the collection of cells for molecular analysis

sometimes caused parts of the trophoblast cells to seep through the fracture during the culture to the blastocyst stage (representative example is illustrated in Fig. 1a). These cells interrupted the removal of the ZP using pronase or tyrode's solution. In order to overcome this problem in some cases the ZP was removed mechanically or alternatively the blastocyst was plated on MEFs without removing the ZP and spontaneously hatched. Another reason for the low success rates may be the method used for ES cell lines isolation. It is possible that other derivation methods such as immunosurgery would have resulted in better isolation rates.

DM is the most common myotonic disorder. In addition to myotonia, this disease may cause multisystem disorders such as progressive muscle wasting, cataracts, cardiac conduction abnormalities, and gonadal atrophy (Mankodi and Thornton, 2002). Although the molecular basis for the disease is known, the processes responsible for the resultant physiological defect are still unknown. Such is the case with the WS disease. The latter is the most common form of inherited congenital deafness (Waardenburg, 1951). Additional symptoms of the disease are pigmentary disturbances of the iris, hair hypopigmentation and dystopia canthorum. Mutations in the PAX3 gene, such as the deletion described in this study, were reported to cause WS (Baldwin et al, 1992; Tassabehji et al, 1992). Although the existence of several *in vitro* models for these diseases (Asher et al, 1996: Tian et al, 2000) availability of human ES cell lines which harbour the DM and WS mutations may have beneficial contribution to the study of these diseases. The pluripotency and immortality of hES cells may be utilized for the development of research models for these diseases. DM and WS diseases affect several systems which make the research on the mechanisms guiding these diseases difficult. The ability of ES cells to differentiate into any cell type of the adult human body can be highly important for revealing the processes affecting each system. Directed differentiation systems for human ES cells carrying these

genetic diseases, into cardiomyocytes, stratified muscle (for DM), nerve cells, and pigment producing cells (for WS) for example, may prove invaluable for better understanding of the diseases pathogenesis. For some of these differentiation models, directing protocols for human ES already exist (Xu et al, 2002; Mummery et al, 2002; Reubinoff et al, 2001; Zhang et al, 2001). These differentiation models can also contribute to the development of drug testing *in vitro* methods for these diseases. Additional benefits may rise from utilizing these ES cell lines for the research on the role of PAX3 gene in nerve early development or the evolution of the $(CTG)_n$ repeats during continuous culture of ES cells.

Gene therapy is often based on targeted correction, using small fragments of a corrected region of the gene (Colosimo et al, 2001). The availability of human ES cell lines which were found to be carriers of cystic fibrosis W1282X mutation and metachromatic leukodystrophy disease mutation enables the development of targeted correction models for these mutations.

The availability of human ES cell lines which harbour genetic defects will allow the monitoring of the expression of these diseases during differentiation. Such a process may be highly effective in revealing the mechanisms responsible for these diseases phenotype and will facilitate the establishment of research models used for development of drugs or gene therapy designed to treat them.

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Figure legends

Fig. 1. Human ES cell lines derivation.. (*A*) Day six expanded blastocyst resulting from an embryo after PGD. Note that part of the trophoectoderm layer sips through the drill in the zona pellucida. This embryo was used for the derivation of the WS1 line. Bar = 30 μM (***B***) ICM outgrowth of the DM1 ES cell line six days post plating the blastocyst in whole on MEFs, The arrow is marking the growing ICM area. Bar = 45 μM. (*C*) Colony of DM1 cell line at passage five growing with MEFs. Bar = 45 μM. (***D***) Undifferentiated cells of the SW1 ES cell line at passage 24. Note the typical spaces between the cells. Bar = 15 μM.

Fig. 2. Molecular analysis of the resultant lines. (*A*) WS PCR products separation on a 3% nusieve agarose (3:1). The product of the affected parent is marked by **A**, example from normal person DNA PCR product is marked by **N** and the PCR product resultant from the WS ES cell line is marked by **L**. The WS line demonstrated the same expression pattern of the affected parent. (***B***) Silver staining of the PCR product of the DM line. PCR products of affected and normal persons were used as controls, and various sizes of $(CTG)_n$ repeats were used as size markers.

Fig. 3. Fluorescent immunostaining for undifferentiated-cells specific surface markers. SW colony visualized with light microscopy (*A*), the same colony, passively stained with SSEA4 (***B***), additional WS colonies visualized with light microscopy (*C*) and passively stained with TRA-1-6 (***D***), another WS line colony visualized with light microscopy (***E***) and stained with TRA-1-81(*F*). Bar = 50 μM.

Fig. 4. Histological section of EBs formed in suspension by the affected cell lines. EB section formed by the DM line (*A*) and by the WS line (***B***). H&E staining. Bar = 60 μM

and 30 μM respectively. Differentiating cells within these EBs stained with nestin (ectoderm) (*C*), insulin (endoderm)(***D***), troponin (mesoderm)(*E*) and (*F*). Bar = 6 μM.

Fig. 5. PCR analysis for representative gene markers of the embryonic germ layers. ***Line 1.*** Undifferentiated cells from the DM line at passage 34. ***Line 2.*** Undifferentiated cells from the WS line at passage 41. ***Line 3.*** EBs formed by the WS line at passage 40. ***Line 4.*** EBs formed by the DM line at passages 30 and 34. ***Line 5.*** RT mix as negative control.

Fig. 6. *In vivo* differentiation of ES cell lines DM and WS in teratomas. (*A*) Teratomas section formed by the WS line which includes secretory epithelium rich in goblet cells (endoderm) and stratified epithelium (ectoderm). Bar = 60 μm (***B***) Teratomas section formed by the WS ES cell line which includes developing bone tissue containing developing bone marrow (mesoderm). Bar = 20 μm (*C*) Developing bone tissue formed by the DM line. Bar = 30 μm (D) Teratomas section formed by the DM line which includes developing eye-like structure (ectoderm) and epithelium (endoderm). Bar = 60 μm

Table 1: RT-PCR conditions.

Table 1

Gene product (Accession number)	*Forward (F) and reverse (R) primers (5'→3')*	*Reaction Condition*	*Size (bp)*
Oct-4 (S81255)	F: GAGAACAATGAGAACCTTCAGGA R: TTCTGGCGCCGGTTACAGAACCA	30 cycles at 60 °C in 1.5 mM $MgCl_2$	219
Albumin (AF542069)	F: TGCTTGAATGTGCTGATGACAGGG R: AAGGCAAGTCAGCAGCCATCTCAT	35 cycles at 60 °C in 1.5 mM $MgCl_2$	302
α-fetoprotein (BC027881)	F: GCTGGATTGTCTGCAGGATGGGGAA R: TCCCCTGAAGAAAATTGGTTAAAAT	30 cycles at 60 °C in 1.5 mM $MgCl_2$	216
NF-68KD (AY156690)	F: GAGTGAAATGGCACGATACCTA R: TTTCCTCTCCTTCTTCACCTTC	30 cycles at 60 °C in 2 mM $MgCl_2$	473
α-cardiac actin (NM_005159)	F: GGAGTTATGGTGGGTATGGGTC R: AGTGGTGACAAAGGAGTAGCCA	35 cycles at 65 °C in 2 mM $MgCl_2$	486
β - Actin (NM_001101)	F:ATCTGGCACCACACCTTCTACAATGAGCTGCG R:CGTCATACTCCTGCTTGCTGATCCACATCTGC	35 cycles at 62 °C in 1.5 mM $MgCl_2$	838

Table 2 Developed blastocysts from donated PGD embryos

Total embryos	Degeneration	Arrested	*Compacted	*Early blast	Blastocysts	Expended blastocyst	Cell lines
76	21	9	8	7	26	5	5

* These embryos stopped to develop beyond the mention stage six days after fertilization.

The arrested and degenerate embryos, developed to the 8-10 cell stage, go through PGD and than stopped to develop.

Claims:

1. Stem cells or embryonic bodies carrying a defective gene.

2. Tissues or organs developed from the stem cells or embryonic bodies of claim 1.

3. The use of the stem cells or embryonic bodies of claim 1 or the tissues or organs of claim 2 in in vitro or in vivo experimental set up as a model for developing cures for genetic diseases.

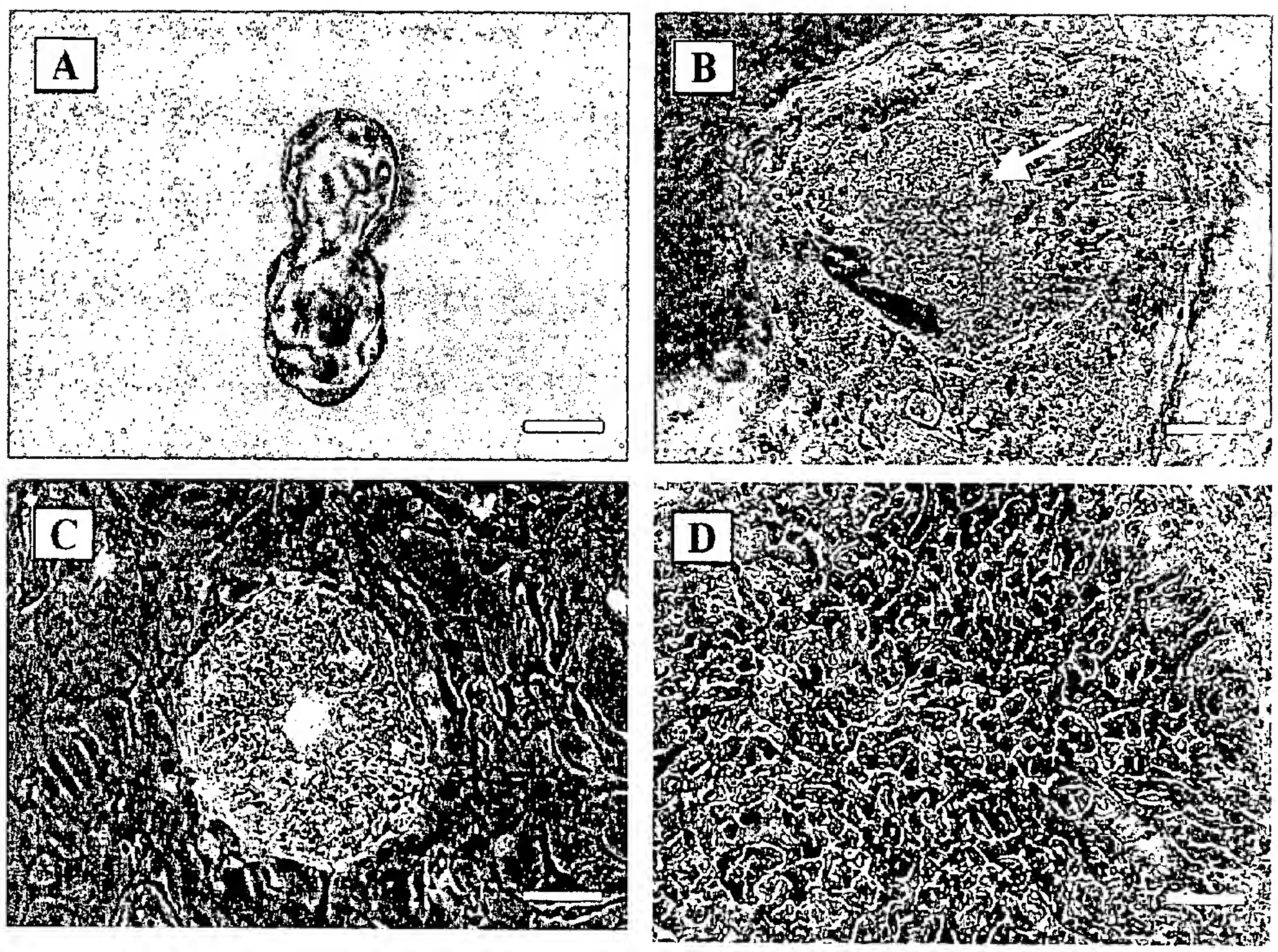

Fig. 1

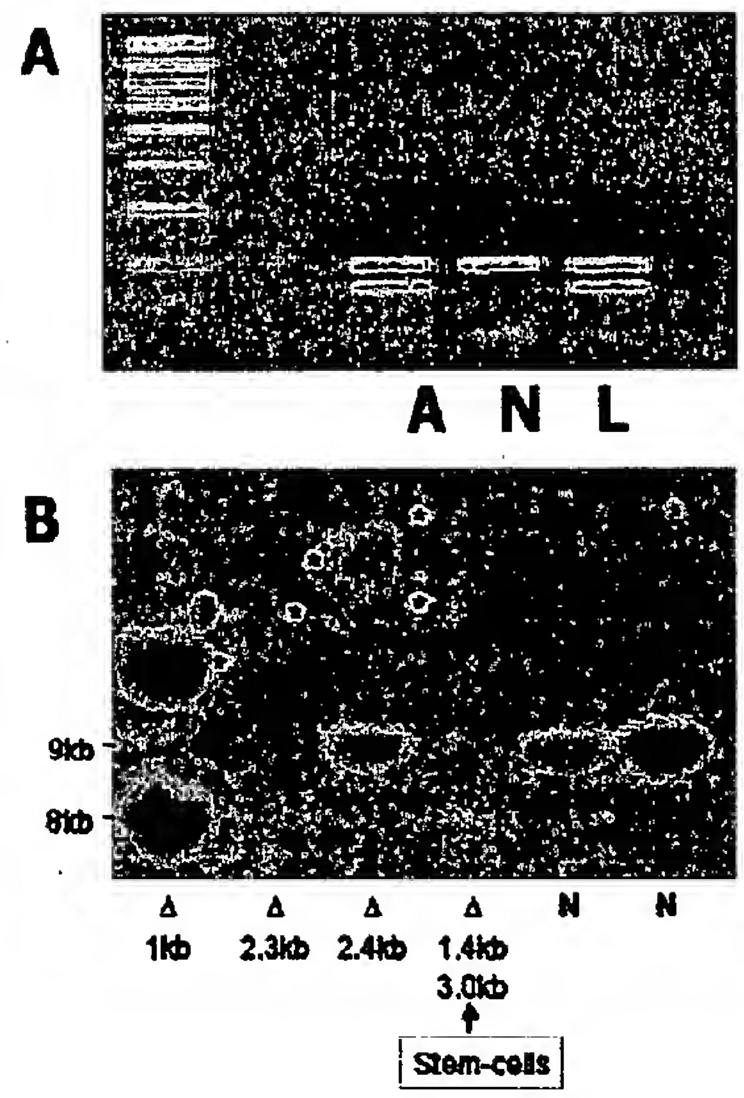

Fig. 2

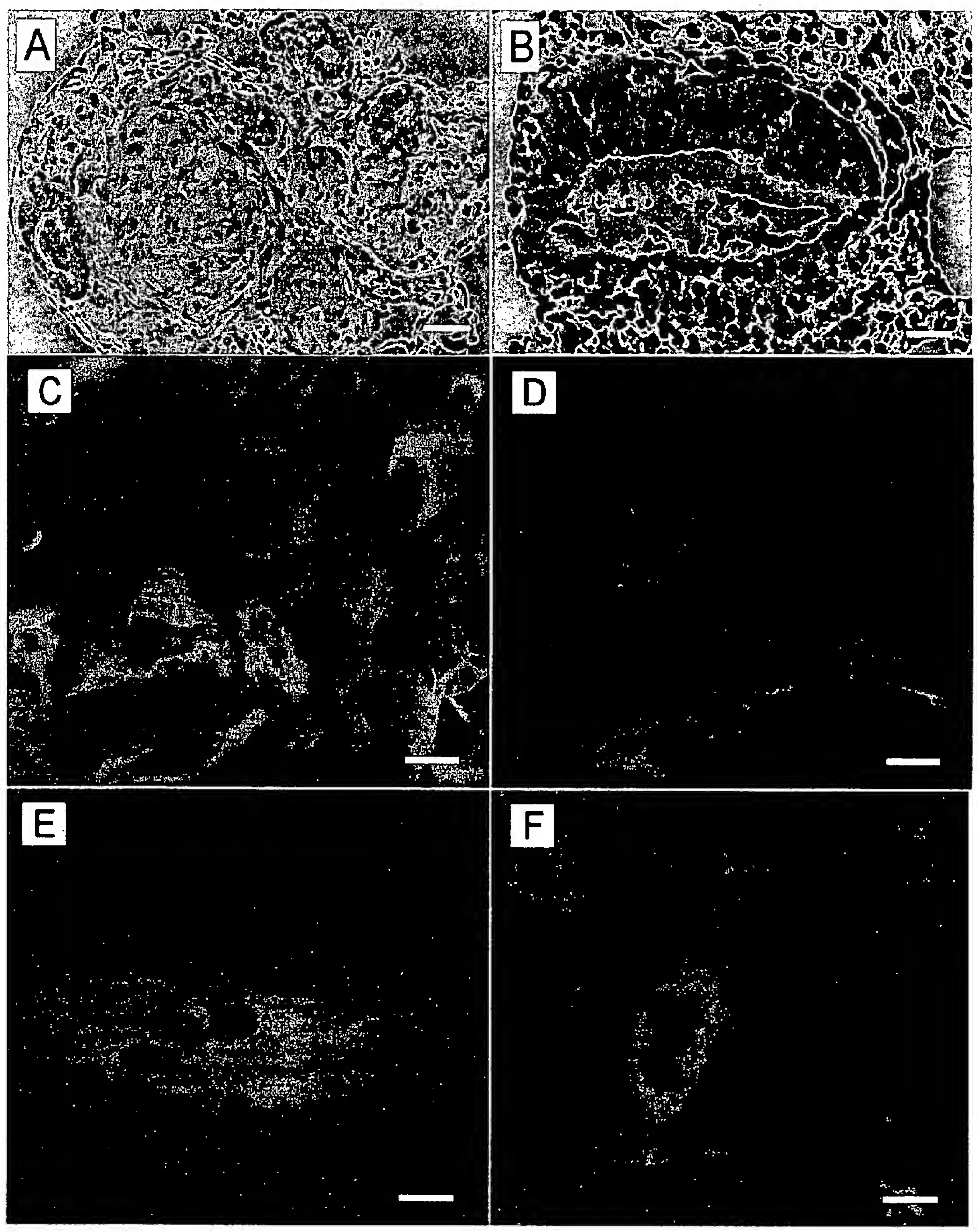

Fig. 4

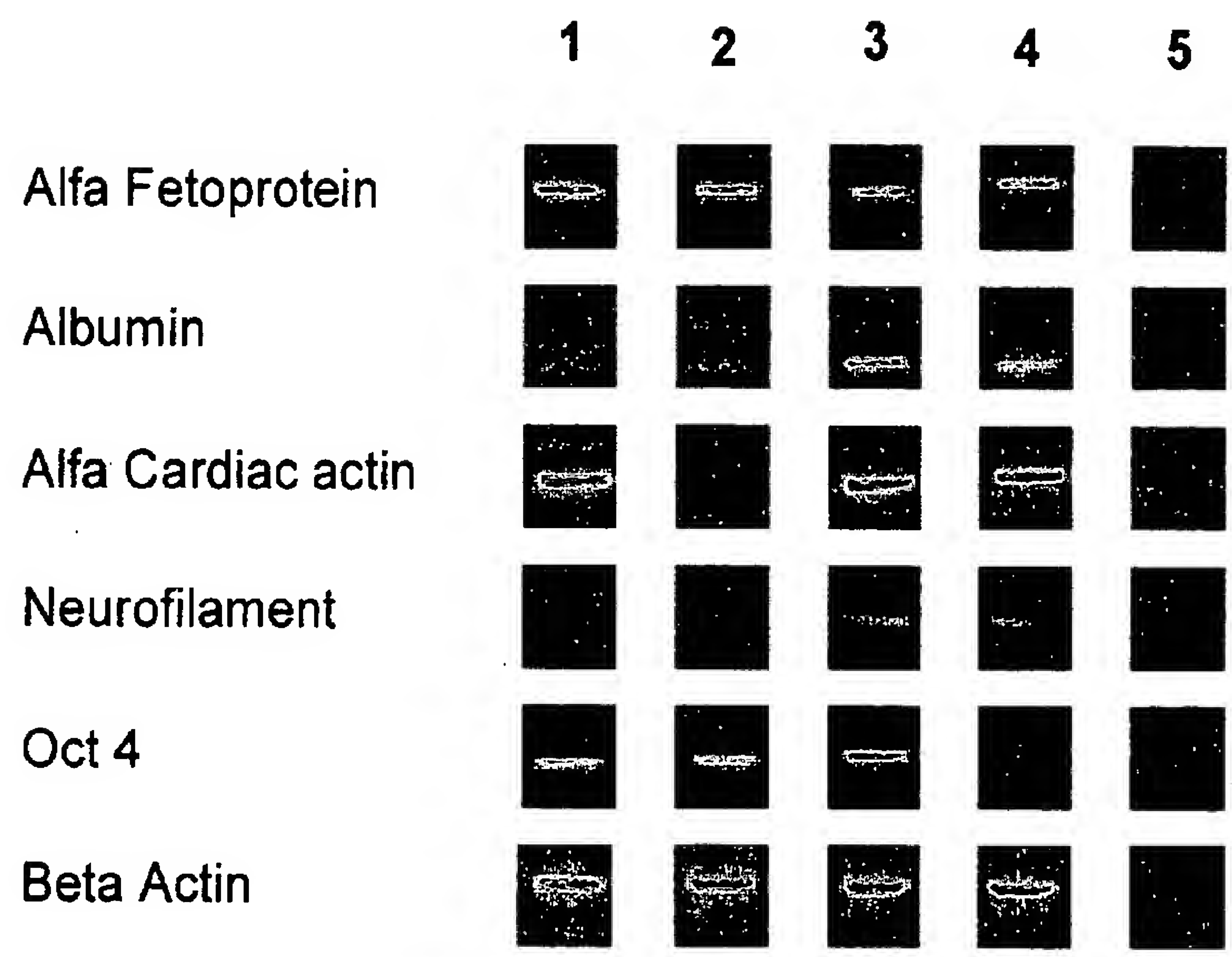
1
2
3
4
5
Alfa Fetoprotein
Albumin
Alfa Cardiac actin
Neurofilament
Oct 4
Beta Actin

Fig. 5

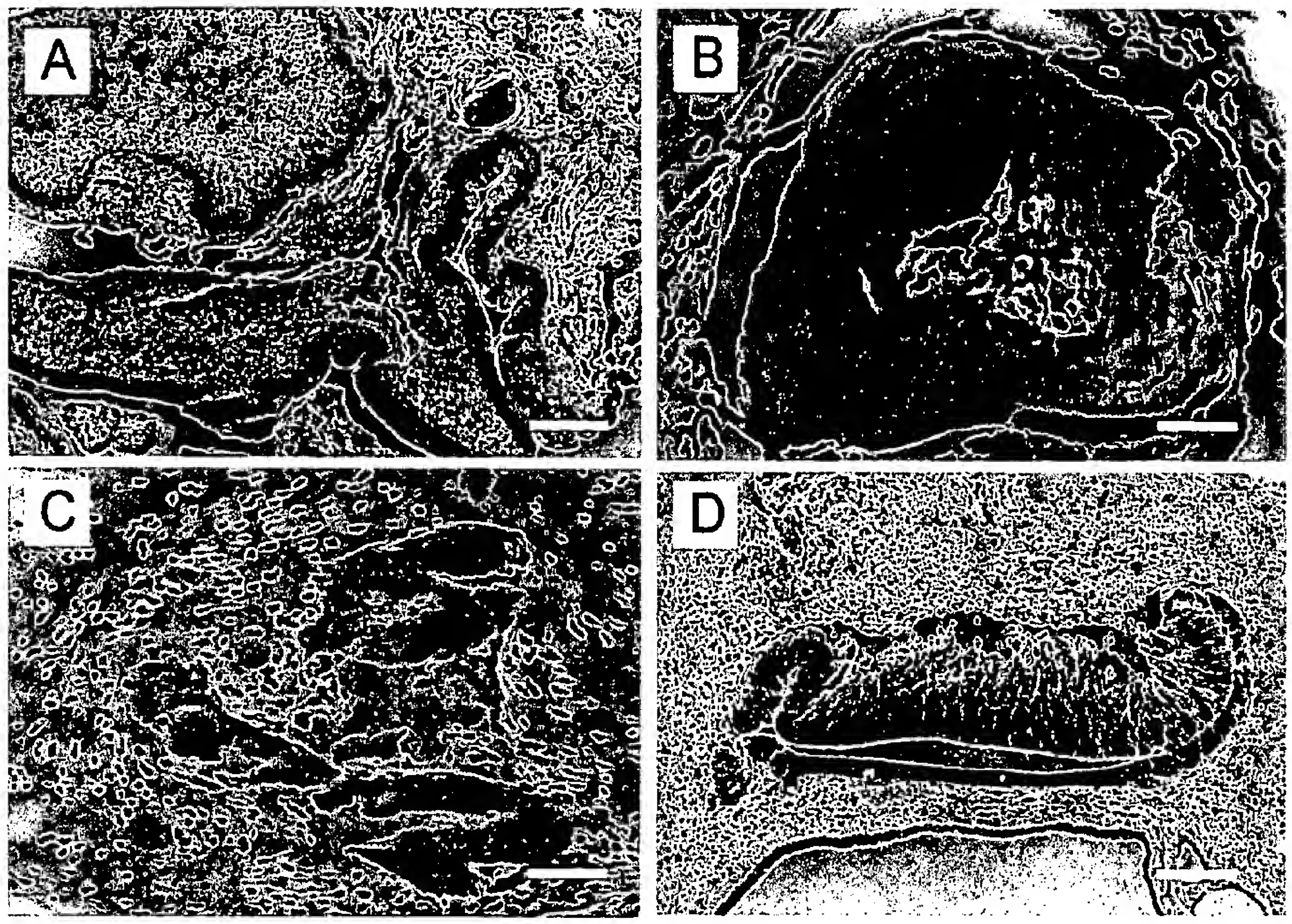

Fig. 6